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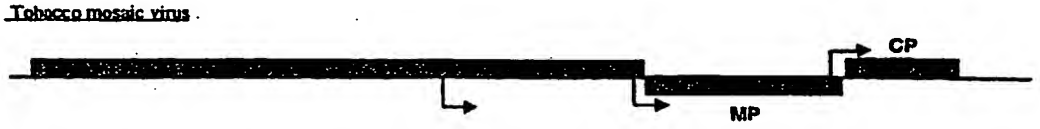

(57) Abstract

The invention provides methods and means for the identification of genes involved in the determination of a plant trait or for the identification encoded by a nucleic acid comprising a determined nucleotide sequence. The invention also provides kits comprising viral RNA vectors derived from satellite viruses and corresponding helper viruses for the introduction of inhibitory RNA into plant cells and plants.

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Methods and means for delivering inhibitory RNA to plants and applications thereof

Field of the invention:

This invention relates to the field of functional genomics in plants, more particularly it relates to methods for the further identification and isolation of a nucleic acid with a nucleotide sequence of interest in a collection of preselected nucleic acid sequences correlated with a particular trait, preferably an agronomical important trait, using a kit of viral RNA vectors which allow systemic spread of all components of the kit in a plant, wherein one of the viral RNA vectors comprises a library of gene-silencing constructs for the preselected nucleic acid sequences. The invention also relates to a method for modulating, preferably reducing, particularly eliminating the expression of a selected nucleic acid sequence, using the viral vector kit, whereby one of the vectors comprises a gene-silencing construct for the selected nucleic acid sequence. The latter method may be used for validating the function of a nucleic acid sequence whose expression is correlated with the presence or absence of a specific trait in plants, but with otherwise unknown function. Preferably, one of the viral RNA vector components of the kit is a vector derived from a satellite virus.

Background art

The recent, rapid expansion of available nucleic acid sequence information has necessitated the development of methods for identifying the function of nucleic acid sequences, particularly transcribed nucleic acid sequences such as expressed sequence tags, with unknown function, in an efficient and labor-cost effective way.

To identify the role of sequenced nucleic acids from plants of unknown function it is necessary to produce or identify plants in which those nucleic acids are either structurally or functionally inactivated. Plants wherein

expression of both sense and antisense constructs in cells of one plant. The sense and antisense nucleic acids may be in the same transcriptional unit, so that a single RNA transcript that has self-complementarity is generated upon transcription.

Hamilton et al. (1998) describe improved silencing e.g. of tomato ACC-oxidase gene expression using a sense RNA containing two additional upstream inverted copies of its 5' untranslated region.

WO 98/53083 describes constructs and methods for enhancing the inhibition of a target gene within an organism, involving the insertion into the gene silencing vector of an inverted repeat of all or part of a polynucleotide region within the vector.

It should be clear however, that the use of inhibitory RNA as a tool in reversed genetics analysis of gene function via high throughput methods, whereby the inhibitory RNA is generated from gene-silencing constructs which are stably integrated in the genome of transgenic plants, suffers from the same drawbacks as the methods wherein the nucleotide sequences are structurally inactivated.

EP 0 194 809 and US 5,500,360 suggest the use of viral RNA vectors to produce regulatory RNA such as anti-sense RNA.

Initial exploration of the use of viral vectors to deliver inhibitory RNA into cells of plants has been described by Chapman (1991). In this publication, gene silencing constructs comprising nucleotide sequences complementary to the translated region of the GUS gene on a PVX derived viral vector were described. The experiments however, remained inconclusive as to whether gene silencing could be provoked using viral vectors for the production of inhibitory RNA.

WO 93/03161 is directed toward recombinant plant viral nucleic acids and to hosts infected thereby. The non-native nucleic acid sequence which is transcribed may be transcribed as an RNA which is capable of regulating the expression of a phenotypic trait by an anti-sense mechanism.

English et al., 1996 describe the suppression of the accumulation of a viral vector comprising a foreign nucleotide sequence in transgenic plants exhibiting silencing of nuclear genes comprising the same foreign nucleotide sequences, thus linking gene silencing and viral vectors, albeit in a reverse way as envisioned here.

Kumagai et al. 1995 (PNAS 92, 1679-1683) described the inhibition of phytoene desaturase gene by viral delivery of antisense RNA.

WO 95/34668 suggests the use of genetic constructs based on RNA viruses which replicate in the cytoplasm of cells to provide inhibitory RNA, either antisense or co-suppressor (sense) RNA.

Baulcombe et al. (1998) and Ruiz et al. (1998) describe virus-induced gene silencing of the endogenous phytoene desaturase gene (PDS) or of a green fluorescent protein transgene (GFP) in plants, using potato virus X derived vectors carrying inserts homologous to PDS and GFP, respectively. The authors further suggested that virus-induced gene silencing may develop into a novel assay of gene function, by introducing a fragment of the genome of a viral vector and inferring the function of the gene from the symptoms of the infected plants exhibiting gene silencing.

The described methods for identification of the function of a gene with known nucleotide sequence however, have drawbacks and limitations. In the first place, the applicability of the mentioned viral RNA vector based gene silencing methods on larger scale is in practice limited to the identification of genes with essential functions or genes with macroscopically visible phenotypes. Secondly, all methods employ viral vectors which are capable of

autonomous replication in plant cells and cell-to-cell movement , whereby care has to be taken not to inactivate the essential functions required for these functions. This may particularly be a disadvantage when tailoring these methods to the needs of particular plants, such as crop plants, by developing new viral vectors more apt for replication and systemic spread in the plants.

The prior art is thus deficient in the lack of efficient methods for large scale identification of the function of nucleic acids with known nucleotide sequence, or for the isolation of the genes of interest from a pool of genes with known nucleotide sequence, but unknown function.

Brief Description of the figures

Figure 1 is a schematic representation of the viral RNA vectors used in the Examples. MP: movement protein; CP: coat protein; OAS: origin of assembly. The open reading frames are indicated by boxes. Each original viral genome is characterized by a specific pattern.

▩ : tobacco mosaic virus; ▨ tobacco necrosis virus; ▩ satellite tobacco mosaic virus; ■ satellite tobacco necrosis virus.

Summary of the Invention

The invention provides a method for isolating genes involved in the determination of a trait or a phenotype of a plant species, comprising identifying a set of nucleic acids sequences of genes, whose expression is correlated with a trait of interest; creating a library of gene silencing constructs targeted or adapted to the nucleotide sequence of the identified nucleic acids in a viral RNA vector which is capable of replication inside plant cells and optionally, movement between plant cells of a plant; infecting a collection of individual plants of the same plant species with the library of gene silencing constructs whereby each plant is infected with at least one member of the library; identifying a plant wherein the trait or phenotype is altered using an assay adapted to that trait or phenotype: and isolating the gene involved in the determination of the trait or phenotype in the plant species, from the library, based on the nucleotide sequence to which the gene silencing construct in the

identified plant was targeted. Preferably the viral RNA vector is capable of autonomous replication inside plant cells and optionally autonomous movement between plant cells and particularly the viral RNA vector is derived from cowpea mosaic virus.

Alternatively, the viral RNA vector, is derived from a satellite RNA virus, preferably satellite tobacco mosaic virus or satellite tobacco necrosis virus, particularly it further comprises an origin of assembly of tobacco mosaic virus, and is capable of replication inside plant cells and optionally movement between plant cells when the required factors are supplemented *in trans*, preferably by infection with a helper virus, preferably tobacco mosaic virus or a helper virus which is derived from tobacco necrosis virus and comprises a gene encoding a coat protein gene of tobacco mosaic virus, and optionally, a movement protein of tobacco mosaic virus.

Gene-silencing constructs comprised within the viral RNA vector may comprise antisense RNA or sense RNA, preferably they may comprise an inverted repeat. Particularly the gene-silencing constructs comprise complementary stretch of at least 50, preferably at least 100 nucleotides of sense and antisense RNA. Especially preferred are gene-silencing constructs comprising at least two copies of part of the nucleotide sequences of the collection of nucleic acids, the copies being in inverted repeat.

It is another object of the invention to provide a method for the isolation or selection of a nucleic acid with a specific function from a collection of nucleic acids, wherein the collection of nucleic acids is characterized by the fact that variation in the expression pattern of the nucleic acids is correlated with variation in a trait or phenotype of a plant harboring the nucleic acids comprising the steps of creating a library of gene silencing constructs targeted or adapted to the nucleotide sequence of the identified nucleic acids in a viral RNA vector which is capable of replication inside and optionally movement between plant cells; infecting a collection of plants with the library of gene silencing constructs. whereby each plant is infected with one member of the

library; identifying plants with altered trait or phenotype using an assay adapted to the trait or phenotype under investigation; and optionally isolating the nucleic acid with the specific function from the identified plant with altered trait or phenotype.

It is yet another object of the invention to provide a method for determining the function encoded by a nucleic acid comprising a known nucleotide sequence in a plant, comprising the steps of providing a viral RNA vector derived from a satellite RNA virus, comprising a gene-silencing construct targeted to a gene comprising the known nucleotide sequence; infecting or inoculating the plant with the chimaeric viral RNA vector and a corresponding helper virus or helper virus RNA; and identifying an altered trait or phenotype of the co-infected plant.

The invention further provides a method for isolating essential genes from a plant, comprising creating a library of random gene-silencing constructs, preferably by cloning random DNA fragments of the plant or by cloning random cDNA fragments, particularly duplicated cDNA fragments in inverted repeat in a cDNA copy of the viral RNA vector derived from a satellite RNA virus, preferably STMV or STNV, particularly a viral RNA vector comprising an origin of assembly of TMV; infecting a plant with individual members of the library and with a corresponding helper virus, preferably tobacco mosaic virus or a helper virus derived from tobacco necrosis virus and comprising the coat protein of tobacco mosaic virus and optionally the movement protein; identifying plants developing gene-silencing-construct-associated necrosis and optionally isolating the viral RNA vector from the necrotized tissue.

It is yet another object of the invention to provide a method for introduction of inhibitory RNA, preferably sense or antisense RNA, particularly inhibitory RNA comprising an inverted repeat, especially inhibitory RNA comprising a complementary stretch of at least 50. preferably at least 100 nucleotides of sense and antisense RNA, into plant cells, preferably into the cytoplasm of plant cells, comprising introducing into a plant cell, a viral RNA vector

comprising the inhibitory RNA or comprising a chimeric nucleic acid which when transcribed yields the inhibitory RNA, wherein the viral RNA vector is derived from a satellite RNA virus, preferably STMV or STNV, particularly a STMV - derived or STNV derived RNA vector comprising an origin of assembly from tobacco mosaic virus; and introducing into the same plant cell, a corresponding helper virus, preferably tobacco mosaic virus or a chimaeric helper virus derived from tobacco necrosis virus and comprising a coat protein gene of tobacco mosaic virus and optionally, a movement protein encoding gene of tobacco mosaic virus.

The invention further provides a kit for introduction of inhibitory RNA, preferably sense or antisense RNA, particularly inhibitory RNA comprising an inverted repeat, especially inhibitory RNA comprising a complementary stretch of at least 50, preferably at least 100 nucleotides of sense and antisense RNA in the cytoplasm of a plant cell comprising 1) a viral RNA vector derived from a satellite RNA virus, comprising the inhibitory RNA or which comprise a chimeric nucleic acid which when transcribed yields the inhibitory RNA; and 2) a corresponding helper virus.

A particularly preferred kit comprises 1) a viral RNA vector derived from a satellite tobacco mosaic virus, comprising an origin of assembly of tobacco mosaic virus, further comprising or encoding the inhibitory RNA; and 2) a corresponding helper virus derived from a tobacco mosaic virus.

Another particularly preferred kit comprises 1) a viral RNA vector derived from satellite tobacco necrosis virus, especially STNV-2 or STNV-C and comprising an origin of assembly of tobacco mosaic virus further comprising or encoding the inhibitory RNA; and 2) a corresponding helper virus derived from tobacco necrosis virus, particularly TNV-A or TNV-D, which comprises a coat protein gene of tobacco mosaic virus and optionally a movement protein gene of tobacco mosaic virus.

Detailed description of preferred embodiments.

The following definitions apply throughout this application, unless otherwise specified.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

As used herein, "a trait of a plant" indicates a phenotype which is the combined result of the coordinated expression of a number of genes. Typical traits include yield, heterosis, drought-resistance, stress-resistance, high or low temperature-resistance, vigor, seed yield, plant habitat, architecture etc. Typically, a trait of a plant is named after its intended appearance.

A used herein a "phenotype" of a plant refers to any quantitative or qualitative characteristic of that plant, be it morphological (including macroscopic and microscopic characteristics), biochemical (including the presence, absence or concentration of particular metabolites or molecules) functional or other.

The term "gene" means any DNA or RNA fragment comprising a region (the "transcribed region") which is transcribed into a RNA molecule (e.g., a mRNA) in a cell, operably linked to suitable regulatory regions, e.g., a plant-expressible promoter. A gene may thus comprise several operably linked fragments such as a promoter, a 5' leader sequence, a coding region, and a 3' region comprising a polyadenylation site. A plant gene endogenous to a particular plant species or virus (endogenous plant or virus gene) is a gene which is naturally found in that plant species or virus, or which can be introduced in that plant species by breeding techniques such as conventional breeding techniques. A chimeric gene is any gene which is not normally found

in a plant species or, alternatively, any gene in which the promoter is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory region of the gene.

The term "expression of a gene" refers to the process wherein a DNA or RNA region which is operably linked to appropriate regulatory regions, particularly to a promoter, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another nucleic acid or which is capable of being translated into a biologically active polypeptide or protein. A gene is said to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as e.g. an antisense RNA, a ribozyme or a replicative intermediate. A gene is said to encode a protein when the end product of the expression of the gene is a biologically active protein or polypeptide. In addition to the above defined elements, a gene may further comprise elements for cap-independent translation such a an internal ribosome entry sequence or the first and second translation enhancing elements as defined in WO 97/49814.

As used herein the terms "gene-silencing" or "inhibitory" are not to be interpreted as meaning a complete abolishing of the expression of the target gene(s) but also includes any reduction in expression, measured either as a reduction in transcription and/or translation, as a reduction in the accumulation of transcripts or translation products such as proteins, or as a reduction in the phenotypic expression of the target gene.

The term "reduction of phenotypic expression" refers to the comparison of the phenotypic expression of the nucleic acid of interest in the eukaryotic cell in the presence of the inhibitory RNA or gene-silencing constructs of the invention, to the phenotypic expression of the nucleic acid of interest in a similar eukaryotic cell in the absence of the inhibitory RNA or gene-silencing constructs of the invention. The phenotypic expression in the presence of the inhibitory RNA of the invention should thus be lower than the phenotypic expression in absence thereof, preferably be only about 25%, particularly only

about 10%, more particularly only about 5% of the phenotypic expression in absence of the inhibitory RNA, especially the phenotypic expression should be completely inhibited for all practical purposes by the presence of the inhibitory RNA or the gene-silencing construct encoding such an RNA.

A reduction of phenotypic expression of a nucleic acid where the phenotype is a qualitative trait means that in the presence of the inhibitory RNA, the phenotypic trait switches to a different discrete state when compared to a situation in which such inhibitory RNA is absent. A reduction of phenotypic expression of a nucleic acid may thus a.o. be measured as a reduction in transcription of (part of) that nucleic acid or reduction in the level of transcript, a reduction in translation of (part of) that nucleic acid or reduction in the level of translation products, or a reduction of the effect the presence of the transcribed RNA(s) or translated polypeptide(s) have on the eucaryotic cell or the organism, and will ultimately lead to altered phenotypes. It is clear that the reduction in phenotypic expression of a nucleic acid of interest, may be accompanied by or correlated to an increase in a phenotype or trait.

In one embodiment of the invention, a method is provided to identify and isolate genes involved in the determination of a trait or a phenotype of a plant. To this end, nucleic acid sequences are identified whose expression is correlated with the trait and/or phenotype of interest. Methods and means are available in the art for the almost simultaneous identification and/or isolation of a large number, if not the predominant part, of nucleotide sequences whose expression, particularly whose transcription, is influenced subsequent to a stimulus corresponding to the trait to be investigated, in comparison with expression of these nucleotide sequences in a control plant. Such methods include but are not limited to differential display methods, such as the gel - based RNA differential display methods described by (Prahasar et al. 1996) As a result of these methods, a collection of at least partially characterized nucleotide sequences with altered expression in response to a particular stimulus is identified. Typically, however, the application of such methods does not allow to discriminate between genes whose altered expression is

directly caused by the stimulus, and those who are further downstream in the chain of events and are only indirectly influenced by the stimulus and a further selection amongst the obtained collection of nucleotide sequences will be required. Even less do these methods allow to predict whether the inverse relationship also holds, i.e. whether influencing the expression of genes with particular nucleotide sequences, identified in the above mentioned way, also influences the trait of interest. A further validation of the obtained sequences is thus required, and preferably one which immediately verifies the above mentioned inverse relationship. To achieve this goal in a efficient and cost-effective way, a library of gene-silencing constructs may be created in a viral RNA vector which is capable of replication inside plant cells and movement between cells of a plant, so as to assure an efficient systemic spread within a plant which has been infected with a clone of such a library. The created library of gene-silencing constructs comprised within a viral RNA vector is then used to infect a representative number of plants in such a way that at each plant is infected by at least one member (one clone) of the library. The infected plants can than be analyzed to identify those plants which exhibit alterations in the trait under investigation, using an assay which is adapted to the trait under investigation. It is clear that this fine-tuning of assay and trait under investigation may be an important advantage over the existing methods for high throughput analysis, particularly when analyzing traits and/or phenotypes which do not result in macroscopically visible alterations, such as but not limited to modifications in specific metabolic pathways or alterations which are only detectable under specific conditions (e.g. heat, stress, drought-tolerance, pathogen-infection, application of specific herbicides or insecticides etc.).

The subset of nucleic acid sequences may also be identified on the basis of the presence of a particular signature characteristic of a class of proteins, such as but not limited to a kinase-specific domain, a binding motif etc.

The gene-silencing construct may then be isolated from the library or from the plant exhibiting the altered trait or phenotype, and be used to isolate the

corresponding gene based on the nucleotide sequence towards which the gene silencing construct was targeted.

In a preferred embodiment, the viral RNA vector is capable of autonomous replication inside plant cells and autonomous movement between plant cells. Such viral RNA vectors are known in the art and may be based on Potato Virus X as described e.g. in WO 93/03161, WO95/34668, Ruiz et al. (1998)

In a particularly preferred embodiment, the used viral RNA vector is derived from cowpea mosaic virus. Wellink et al. (1998) have described the use of a viral vector derived from this RNA virus for the expression of GFP in plants and demonstrated that the virus and RNA vectors derived thereof have an excellent capacity for spreading throughout an infected plant, particularly *Nicotiana benthamiana*. CPMV is an icosahedral virus with a bipartite RNA genome, consisting of a longer and a shorter RNA. Wellinck et al. have demonstrated that it is possible to incorporate extra genetic information in the shorter RNA, by inserting the GFP coding region in frame into the viral encoded polyprotein. For the purpose of the herein described methods, it is preferred that the inhibitory RNA encoding nucleic acid be inserted downstream of the polyprotein encoding open reading frame.

The inventors have obtained for the first time indications that gene-silencing may be obtained in plant cells such as protoplasts, using a viral vector derived from a satellite virus comprising a β-1,3-glucanase coding region, in a co-infection experiment with a helper virus of the satellite virus

In another preferred embodiment, the viral RNA vector is capable of replication and cell-to-cell movement, only when the required functions are provided *in trans*. Particularly preferred, is the use of a satellite virus derived RNA vector, which can replicate in plant cells and spread throughout the plant, when a corresponding helper virus is present.

As used herein, "a satellite virus" indicates an RNA virus, preferably a single stranded RNA virus, the RNA genome of which is capable of replicating in a plant cell and being encapsidated by coat protein molecules to form a virus particle or virion, only when provided externally with any number of required essential functions therefor. By "externally provided" is meant that such functions are not encoded by the satellite viral genome. Satellite viruses thus depend upon external provision of essential functions, and may lack the capacity to encode functional replicase, movement protein, or other essential functions required to complete their life cycle inside a plant cell. In a natural situation, such essential functions are usually provided by an autonomously replicating virus or so-called helper virus .

Satellite viruses useful for the present invention may include wild type isolates, but also encompassed by this definition are variants which result in reduced or minimal symptoms when infected on a host plant, particularly when co-inoculated with a corresponding helper virus. The definition also includes synthetic satellite viruses such as defective viruses and chimeric satellite viruses.

A "viral RNA vector derived from a satellite virus" should at least include cis elements from a satellite virus which are recognized by an externally provided replicase, and an origin of assembly allowing encapsidation by the provided coat protein. Preferably, the viral RNA vector does not comprise a gene encoding a functional coat protein, particularly it does not comprise the nucleotide sequence which is essentially similar to the nucleotide sequence encoding a coat protein gene. Particularly preferred are viral RNA vectors comprising an origin of assembly recognized by coat protein molecules from a rod-shaped virus, such as tobacco mosaic virus, since rod-shaped viruses do not exhibit the spatial constraints imposed on the size of genome by icosahedral viruses, thus allowing a larger number of additional nucleotides to be incorporated in the viral vector. Conveniently, the viral RNA vector comprises a number of unique or low-occurrence restriction recognition sites.

The use of viral RNA vectors derived from a satellite virus, additionally solves problems associated with the use of viral RNA vectors, such as reducing the size of the vectors, increasing versatility etc.

Particularly suited for the invention are viral RNA vectors derived from satellite tobacco mosaic virus comprising the origin of assembly (OAS) from TMV, preferably comprising the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5443 to the nucleotide at position 5518 or the nucleotide of SEQ ID No 5 from the nucleotide at position 5430 to the nucleotide at position 5505 (such as the nucleotide sequence of SEQ ID No 12) and wherein the coat protein encoding gene has been deleted. Also particularly suited for the invention are viral RNA vectors derived from satellite necrosis vector strain comprising the OAS from TMV and wherein most of the coat protein gene has been deleted. Non-limiting examples of viral RNA vectors, suitable for the invention are described hereinafter.

"A corresponding helper virus" as used herein, indicates those RNA viruses, preferably singe stranded RNA viruses, which can supply the satellite virus or the derived viral RNA vector with the functions required *in trans* by that satellite virus or the derived viral RNA vector, to allow it to replicate in the cytoplasm of plant cells, and spread throughout an infected plant. Typically, corresponding helper viruses will provide the satellite virus or the vector derived thereof with a replicase (RNA dependent RNA polymerase) which recognizes the *cis* sequences present on the satellite virus RNA, and will allow replication of the satellite virus genome or the derived vector. Other proteins which may typically be provided by the helper virus are movement proteins, allowing inter alia, the plasmodesmata-mediated spread of viral particles from cell to cell. For satellite viruses or viral RNA vectors derived thereof which lack a functional coat protein encoding gene, corresponding helper viruses may also provide a functional coat protein. Preferably, the corresponding helper virus will be capable of autonomous systemic spread in an infected plant. However, such a systemic spread seems not to be a prerequisite for efficient gene silencing. Functions required *in trans* for one

particular viral RNA vector may be supplied *in trans* by different corresponding helper viruses.

It is clear that the corresponding helper viruses may be wild type isolates of RNA viruses, preferably single-stranded RNA viruses such as the tobamoviruses or necroviruses. Particularly preferred are rod-shaped RNA viruses such as tobamoviruses including tobacco mosaic virus and the related tobamoviruses such as ribgrass mosaic virus, turnip vein clearing virus, chines rape mosaic virus, oilseed rape mosaic virus.

When TMV can be used as a helper virus, it can also be replaced by one of the closely related tobamoviruses mentioned above, particularly when using the viral vectors in particular plant species.

Also encompassed by the methods and means of the invention are variants of such wild type isolates, preferably variants or mutants which develop minimal symptoms when inoculated on host plants or when co-infected with a corresponding satellite virus or RNA vector derived thereof. Further preferred helper viruses may be variants or mutants of wild type isolates which have an extended host range such as tobamoviruses which can replicate and spread in corn or brassicae.

However, corresponding helper viruses may also be chimeric or hybrid viruses, wherein part of the viral genome has been replaced by a foreign nucleic acid, particularly wherein part of the viral genome has been replaced by a nucleic acid derived from another viral genome, preferably a part comprising a nucleotide sequence encoding a movement protein, or a part comprising a nucleotide sequence encoding a coat protein. E.g. when using a necrovirus such as TNV, it may be advantageous to insert a movement protein encoding region , preferably a movement protein derived from a tobamovirus such as TMV, particularly the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 4903 to the nucleotide at position 5709, so as to ensure spreading of the viral particles beyond the infected leaf. However,

spreading of the helper virus or the viral RNA vector is not essential for efficient inactivation of expression of the target genes throughout the plant, as was found by the inventors. Also when using e.g. a necrovirus such as TNV, it may be further advantageous to replace the coat protein coding region of the necrovirus by a coat protein coding region of a rod-shaped virus, such as TMV, particularly the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5712 to the nucleotide at position 6191. It goes without saying that an appropriate origin of assembly for the substituted coat protein has to be incorporated in the genome of the chimaeric helper virus. In the above described example however, the OAS of TMV is conveniently located within the movement protein coding region. Non-limiting examples of corresponding helper viruses will be described hereinafter.

It should be clear that whenever it is stated that plants are co-infected or infected with a viral RNA vector and a corresponding helper virus, it is equal whether the helper virus is inoculated before, after or simultaneous with the viral RNA vector, provided however that there is a reasonable time limit between infection of the viral RNA vector or the corresponding helper virus.

Alternatively, the required functions *in trans* for the replication and movement of the viral RNA vector may be provided from the expression of chimeric genes, encoding a replicase (RNA dependent RNA polymerase) and/or a movement protein and/or a functional coat protein, integrated in the genome of the test plants.

Preferred kits to deliver inhibitory RNA or gene-silencing constructs to plant cells to be used in the herein disclosed methods comprise a viral RNA vector derived from a satellite RNA virus, particularly from satellite tobacco necrosis vector (STNV) or satellite tobacco mosaic virus (STMV) and a corresponding helper virus, particularly a rod-shaped corresponding helper virus, wherein the viral RNA vector comprises a gene-silencing construct.

In a preferred embodiment the kit comprises a viral RNA vector derived from satellite tobacco necrosis vector, preferably comprising the cis-elements required for replication, particularly comprising the nucleotide sequence of SEQ ID No 3 from the nucleotide at position 1 to the nucleotide at position 32 and the nucleotide sequence of SEQ ID No 3 from the nucleotide at position 738 to the nucleotide at position 1245, wherein an origin of assembly of tobacco mosaic virus has been inserted, preferably comprising the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5443 to the nucleotide at position 5518 or comprising the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 5430 to the nucleotide at position 5505, or comprising the nucleotide sequence of SEQ ID No 12 and wherein said helper virus is derived from tobacco necrosis virus, preferably with a nucleotide sequence of SEQ ID No 1, and comprises a gene encoding the movement protein of tobacco mosaic virus, preferably with the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 4903 to the nucleotide at position 5709 or with the nucleotide sequence of SEQ ID No 15 from the nucleotide at position 479 to the nucleotide at position 1285 and a gene encoding the coat protein of tobacco mosaic virus, preferably with the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5712 to the nucleotide at position 6191 or with the nucleotide sequence of SEQ ID No 15 from the nucleotide at position 1288 to the nucleotide at position 1767.

Preferred combinations are those kits wherein the viral RNA vector is derived from STNV-1 or STNV-2 strains (as disclosed by Ysebaert et al. 1980; Genbank Accession number M10388 or Danthinne et al., 1991 Genbank Accession M64479) and the helper virus is TNV-A (Meulewaeter et al 1990, SEQ ID No 1). Other preferred combinations are those kits wherein the viral RNA vector is derived from STNV-C (Bringloe et al. (1998); Genbank Accession Nr AJ000898) and the corresponding helper virus is TNV-D (Coutts et al. (1991); Genbank Accession Nr D00942).

In another particularly preferred embodiment the kit comprises a viral RNA vector derived from satellite tobacco mosaic virus, preferably comprising the

cis-elements required for replication, particularly comprising the nucleotide sequence of SEQ ID No 4 from the nucleotide at position 1 to the nucleotide at position 197 and the nucleotide sequence of SEQ ID No 4 from the nucleotide at position 604 to the nucleotide at position 1058 or comprising the nucleotide sequence of SEQ ID No 13 and the nucleotide sequence of SEQ ID No 14; and further comprising an origin of assembly of tobacco mosaic virus, preferably comprising the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5443 to the nucleotide at position 5518 or comprising the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 5430 to the nucleotide at position 5505 or comprising the nucleotide sequence from SEQ ID No 12, and wherein said corresponding helper virus is a tobacco mosaic virus, particularly TMV-U1 (SEQ ID No 2) or TMV-U2 (SEQ ID No 5).

It will be clear to the person skilled in the art that viral RNA vectors may be generated conveniently by *in vitro* transcription methods from cDNA copies of the viral RNA. Likewise, infectious viral RNA for the corresponding helper viruses may be generated from cDNA copies of their genome. Libraries, viral vectors and corresponding helper viruses may also be maintained by replication in plant cells.

Methods to infect or inoculate plants and plant cells with viral RNA vectors, helper viruses and libraries comprised within viral RNA vectors are well within in the realm of the person skilled in the art and may be performed according to the methods described in Walkey (1985).

In one embodiment of the methods of the invention, plants are inoculated, e.g. with a solution containing the libraries of gene-silencing constructs in a viral vector, or with a solution containing a mixture of gene-silencing constructs in a viral vector and corresponding helper virus. The solution may further contain additional compounds to improve inoculation and infection of the plants, such as, but not limited to abrasives, adherents, tensio-active products and the like.

Plants may be infected during different developmental stages, in order to maximize the phenotype under investigation. Also different parts of plants may be inoculated to optimize observation of the expected phenotype.

Although not intending to limit the scope of the invention to a particular mode of action, it is thought that the inhibitory RNA comprised within the viral RNA vector can exercise its inhibiting effect, provided there is a balance between RNA encapsidated in a virion and free RNA. It is thought that the balance between encapsidated and free RNA may be influenced by varying the sequence and position of an origin of assembly within the viral RNA vector. However, the gene-silencing effect may be amplified by placing the inhibitory RNA encoding nucleic acid under control of a viral promoter, preferably a coat protein promoter, or a subgenomic promoter so that during the life cycle of the virus additional inhibitory RNA is generated or transcribed.

"Gene-silencing constructs" as used herein is to be interpreted as a nucleic acid, which when transcribed yield "inhibitory RNA" comprising or consisting of sense RNA or antisense RNA, or a combination of both comprising a nucleotide sequence which has at least 75%, preferably at least 80%, particularly at least 85%, more particularly at least 90%, especially at least 95% sequence identity with or is identical to the nucleotide sequence whose expression is to be suppressed, or its complement. Further, the nucleotide sequence of the sense or antisense region should preferably be at least about 100 nucleotides in length, more preferably at least about 250 nucleotides, particularly at least about 500 nucleotides but may extend to the full length of the coding region of the gene whose expression is to be reduced.

For practical purposes in the application of the methods for high throughput screening or validation, the gene-silencing construct may be identical in sequence and length to the target nucleic acids, or they may be exactly complementary in sequence and identical in length to the target nucleic acids.

For the purpose of this invention the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Wilbur and Lipmann algorithm (Wilbur and Lipmann ,1983) using a window-size of 20 nucleotides or amino acids, a word length of 2 amino acids, and a gap penalty of 4. Computer-assisted analysis and interpretation of sequence data, including sequence alignment as described above, can be conveniently performed using commercially available software packages such as the programs of the Intelligenetics™ Suite (Intelligenetics Inc., CA) or the GCG Wisconsin Package.

It is clear for the person skilled in the art that the gene-silencing constructs may comprise at the same time sense and anti-sense RNA targeted towards the same nucleotide sequence whose expression is to be reduced. Preferably, the sense and antisense RNA are at least partly complementary to each other and capable of forming a stem-loop structure, since such a configuration has been shown to increase the efficiency of gene-silencing, both in occurrence and level of gene-silencing (Waterhouse et al. 1998). In the most straightforward embodiment, at least part of the target nucleic acid, preferably the complete target nucleic acid, is cloned in duplicated form, whereby the two copies are in inverted repeat, preferably separated by an unrelated spacer nucleotide sequence.

The invention also aims at providing the herein described kits in their different embodiments. It is also an object of the invention to provide the kits comprising the helper viruses and viral RNA vectors described without the gene-silencing constructs or inhibitory RNA as well as their cDNA copies, whereby the cDNA copies are under control of a promoter (which can be used in *in vitro* transcription methods available in the art) such as but not limited to

the promoters recognized by single subunit bacteriophage polymerase promoters (T7, T3, SP6 RNA polymerase specific promoters and the like).

The invention further relates to a method for identifying genes which are essential in plants comprising the following steps:

a) A library of random gene-silencing constructs specific for the plant is created using a viral RNA vector which is derived from a satellite RNA virus, as herein defined including all its preferred embodiments. Preferably, the library is created in a cDNA copy of the viral vector and may be generated by inserting random DNA sequences, preferably at least about 100 nucleotides in length, particularly at least about 500 nucleotides in length. The random DNA sequences may be obtained from total DNA of a plant or may represent a subset of the genome of a plant, such as DNA derived from organelles (plastids, chloroplasts, mitochondria etc.) Alternatively, the library may be created by inserting cDNAs generated by reverse transcriptase from RNA, preferably mRNA obtained from said plant. The library may be normalized, e.g. as described in Takayuki et al. (1995). The library should preferably be large enough in size, i.e. contain a sufficient number of independent clones to cover the genome of the plant, according to the standards known in the art. In a preferred embodiment, the library may contain duplication of the inserted nucleic acid whereby the copies are in inverted repeat. The inserted nucleic acid may be cloned downstream of a viral promoter such as, but not limited to a coat protein gene promoter or a subgenomic promoter. It will further be clear to the person skilled in the art that the relative orientation of the inserted nucleic acid, in relation to the RNA vector is only of limited importance since either sense or antisense inhibitory RNA will be generated.

b) Assay plants are infected with individual members of the library and also with a corresponding helper virus. Infection may proceed according to any of the methods mentioned herein. Clearly, the DNA copy of the library should be converted into an RNA copy according to any of the methods described herein, preferably prior to the infection of the assay plants.

c) Plants developing a gene-silencing-construct-associated phenotype are identified. As used herein, a "gene-silencing-construct-associated phenotype" is meant to indicate a phenotype which is not observed when performing a mock inoculation with a viral RNA vector without gene-silencing construct, in combination with a corresponding helper virus on a similar plant. Preferred phenotypes comprise chlorosis, necrosis or any phenotype, preferably a morphological phenotype indicating that the infected tissue is inhibited or dying or deteriorating.

d) Optionally , isolating the viral RNA vector from the tissue exhibiting the gene-silencing-construct-associated phenotype according to methods available in the art for isolation of virus. Preferably, the isolated viral RNA vector comprising the gene-silencing construct of interest should be re-assayed on fresh plants to confirm the observed phenotype. The viral RNA vector can of course also be recovered from the library if the infection of the plants was performed in an identity -preserving way.

e) The gene silencing construct or the nucleotide sequence information thereof, may then be used to recover the corresponding genomic or cDNA clone using methods available in the art (hybridization, PCR etc.)

As defined herein "essential genes" of a plant, are those genes which are necessary during the normal development of a plant. As defined, essential genes may be essential for normal development only in particular developmental stages, or only in particular tissues or organs, such as e.g. flowers. Typically, inhibition of the expression of essential genes may have a lethal effect on a plant or part of a plant. Preferred essential genes are those genes which result in retardation or dying of seedlings when inhibited.

It will be clear for the person skilled in the art that if the inhibition of the target nucleic acid results in a dominant effect, as is the case for inhibition of the expression of essential genes, the described methods may be performed using infection of more than one viral RNA vector comprising a gene-silencing construct per plant. Care has to be taken to not dilute the phenotypic effect too much by infecting a too large number of different viral RNA vectors

comprising differing gene-silencing constructs on the same plant. It is thought that optimally any number between one and five different inhibitory RNAs may be introduced in one plant cell.

In yet another embodiment of the invention, a method is provided for determining the function encoded by a nucleic acid comprising a known nucleotide sequence. This nucleotide sequence may have been obtained e.g. from a genome sequencing program, including expressed sequence tags sequencing programs. In order to unravel the function of that sequence, a gene-silencing construct or inhibitory RNA targeted towards said nucleotide sequence, as described in all its embodiments herein, may be introduced into a viral RNA vector derived from a satellite virus, as described herein, and used to inoculate a plant or being introduced into a plant cell, particularly into a protoplast, together with a corresponding helper virus, as described herein.

A large number of the embodiments described herein thus relate to a method for the introduction of inhibitory RNA in plant cells, comprising the steps of :

a.) introducing into a plant cell, a viral RNA vector comprising inhibitory RNA or comprising a chimeric nucleic acid which when transcribed yields the inhibitory RNA, wherein the viral RNA vector is derived from a satellite RNA virus; and

b.) introducing into the same plant cell, a corresponding helper virus.

The methods of the invention can be applied to essentially all plants for which viral vector and/or corresponding helper viruses are available. The methods of the invention are thought to be particularly suited for Nicotina spp, particularly N. tabacum, N. sylvestris, N. benthamiana, and other Solanacea, rice (Oryza sativa) corn (Zea Mays), Brassica spp. , cotton (Gossypum hirsutum), wheat, Arabidopsis spp., Petunia spp.

Also envisioned by the present invention are methods for developing an agronomically useful product, such as a herbicide or a transgenic plant using the herein described methods and means, further comprising the steps of

inserting a nucleic acid, involved in the determination of a particular plant trait, isolated by the methods of the invention, preferably under control of a foreign plant-expressible promoter, particularly under control of a controllable plant-expressible promoter into the genome of a plant , particularly a crop plant. When essential genes have been identified according to the methods described herein, these essential genes or their encoded gene products, particularly the encoded proteins may be used in *in vitro* assays to identify compounds inhibiting the activity, particularly the enzymatic activity, which may be used as herbicides. Alternatively, a viral RNA vector encoding gene-silencing constructs targeted towards essential genes may be used as herbicidal compounds.

The following non-limiting Examples describe the construction of viral RNA vectors derived from satellite viruses, and uses thereof. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No 1: nucleotide sequence of the genome of TNV-A

SEQ ID No 2: nucleotide sequence of the genome of TMV-U1 (Genbank Accession Nr V01408).

SEQ ID No 3: nucleotide sequence of the genome of STNV-2

SEQ ID No 4: nucleotide sequence of the genome of STMV (Genbank accession Nr. M25782).

SEQ ID No 5: nucleotide sequence of the genome of TMV-U2 (Genbank Accession Nr M34077).

SEQ ID No 6: nucleotide sequence of the tomato phytoene desaturase (*pds*) encoding cDNA (Genbank Accession Nr. X59948).

SEQ ID No 7: nucleotide sequence of the tobacco nitrate reductase (*nia*-2) encoding cDNA (Genbank Accession Nr. X14059).

SEQ ID No 8: nucleotide sequence of the tobacco nitrite reductase (*nir*-1) encoding cDNA (Genbank Accession Nr. X66145).

SEQ ID No 9: nucleotide sequence of the β-1,3-glucanase(*gn*-1) encoding cDNA of *Nicotiana plumbagenifolia*.

SEQ ID No 10: nucleotide sequence of a green fluorescent protein (*gfp*) encoding region.

SEQ ID No 11: nucleotide sequence of a β-glucuronidase (*gus*) encoding region.

SEQ ID No 12: nucleotide sequence of an origin of assembly of a TMV-U2 strain.

SEQ ID No 13: nucleotide sequence of the leader sequence of a STMV strain

SEQ ID No 14: nucleotide sequence of the trailer sequence of a STMV strain.

SEQ ID No 15: nucleotide sequence of part of the genome of a TMV-U2 strain comprising movement protein and coat protein genes.

Examples

Example I : construction of the viral RNA vector kits

A plasmid vector for the synthesis of an infective hybrid TMV/TNV helper virus RNA is made using the following operationally linked elements:

- A T7 RNA polymerase promoter
- A nucleic acid comprising the nucleotide sequence from the nucleotides 1 to 2234 of TNV-A (nt 1 to 2234 of SEQ ID No 1), wherein the AUG codon at nucleotides 2218-2220 mutated to a different codon
- A nucleic acid comprising the nucleotide sequence encoding the open reading frame for the movement protein of TMV-U1 (nt 4903-5709 of Genbank Accession Number V01408 or nt 4903-5709 of SEQ ID No 2 or nt 479-1285 of SEQ ID No 12)
- A nucleic acid comprising the nucleotide sequence from the nucleotides 2235 to 2612 of TNV-A (SEQ ID No 1)
- A nucleic acid comprising the nucleotide sequence encoding the open reading frame for the coat protein of TMV-U1 (nt 5712-6191 of Genbank Accession Number V01408 or nt 5712-6191 of SEQ ID No 2 or nt 1288-1767 of SEQ ID No 15)
- A sequence comprising nucleotide 3444 to 3684 of TNV-A (nt 3444 to 3684 of SEQ ID No 1)

A plasmid vector for the synthesis of an infective hybrid TMV/STNV viral vector RNA is made using the following operationally linked elements:

- A T7 RNA polymerase promoter
- A nucleic acid comprising the nucleotide sequence from nucleotide 1 to 32 of STNV-2 (nt 1 to 32 of SEQ ID No 3)
- A nucleic acid comprising the origin of assembly (OAS) of TMV-U1 (nt. 5443-5518 of Genbank Accession Number V01408 or nt 5443-5518 of SEQ ID No 2 or nt 1018 to 1094 of SEQ ID No 15)
- A nucleic acid comprising the nucleotide sequence from nucleotide 738 to 1245 of STNV-2 (nt 738 to 1245 of SEQ ID No 3)

A plasmid vector for the synthesis of an infective hybrid TMV/STMV viral vector RNA is made using the following operationally linked elements:

- A T7 RNA polymerase promoter
- A nucleic acid comprising the nucleotide sequence from nucleotide 1 to 197 of STMV (Genbank Accession Number M25782; nt 1 to 197 of SEQ ID No 4) or the nucleotide sequence of SEQ ID No 13.
- A nucleic acid comprising the OAS of TMV-U1 (nt. 5443-5518 of Genbank Accession Number V01408; nt 5443 to 5518 of SEQ ID No 2; nt 1019-1094 of SEQ ID No 15) or of TMV-U2 (nt 5430-5505 of Genbank Accession Number M34077; nt 5430-5505 of SEQ ID No 5) such as the nucleotide sequence of SEQ ID No 12.
- A nucleic acid comprising the nucleotide sequence from nucleotide 604 to 1058 of STMV (Genbank Accession Number M25782; nt 604 to 1058 of SEQ ID No 4) or comprising the nucleotide sequence of SEQ ID No 14.

Example 2. Feasibility demonstration using known endogenes or transgenes.

To demonstrate the feasibility of the use of the viral kits described sub example 1 for functional knockout of specific endo- or transgenes in *Nicotiana* plants, one of the following DNA fragments is inserted in the TMV/STNV or TMV/STMV hybrid vector, immediately upstream or downstream of the TMV OAS:

- a fragment of the tomato pythoene desaturase (*pds*) cDNA (comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948)
- a fragment of the tobacco nitrate reductase (*nia-2*) cDNA (comprising nucleotides 1103 to 2114 or nucleotides 5169 to 6497 of SEQ ID No 7 or Genbank Accession Number X14059)
- a fragment of the tobacco nitrite reductase (*nir-1*) cDNA (comprising nucleotide 650-1212 of SEQ ID No 8 or Genbank Accession Number X66145)

- a fragment of the β-1,3-glucanase (*gn-1*) cDNA of *Nicotiana plumbaginifolia* (SEQ ID No 9 or Genbank Accession Number X07280)
- a fragment comprising a nucleotide sequence from a green fluorescent protein (*gfp*) coding region (SEQ ID No 10)
- a fragment comprising a nucleotide sequence from nucleotide 1 to 600 of the β-glucuronidase (*gus*) coding region (SEQ ID No 11)

Infective chimeric transcripts are synthesized *in vitro*, using T7 RNA polymerase with the linearized plasmid DNAs of the described vectors as templates.

The TMV/STNV RNAs are mechanically inoculated on leaves of *Nicotiana benthamiana* or *Nicotiana tabacum* plants together with the TMV/TNV RNA, whereas the TMV/STMV RNAs are inoculated together with TMV-U2 virus particles or viral RNA.

The infected plants are scored for phenotypes, virus accumulation, and suppression of the homologous plant gene between 1 and 4 weeks after inoculation.

Plants infected with vectors containing the *pds* cDNA show a bleaching phenotype on infected leaves and silencing of the endogenous *pds* transcript.

Plants infected with vectors containing the *nia-2* or *nir-1* cDNA show a chlorotic phenotype on infected leaves and silencing of the endogenous *nia-2* or *nir-1* transcript, respectively.

Plants infected with vectors containing the g*n-1* cDNA show silencing of the endogenous basic -1,3-glucanase transcript.

Upon infection with vectors containing the *gfp* sequence, transgenic plants that normally express a *gfp* transgene show silencing of the *gfp* transgene transcript and suppression of GFP fluorescence.

Upon infection with vectors containing the *gus* sequence, transgenic plants that normally express a *gus* transgene show silencing of the *gus* transgene transcript and suppression of GUS activity.

Example 3: Inactivation of phytoene desaturase in Nicotiana benthamiana using a TNV/STNV hybrid vector system.

A TNV/STNV hybrid vector system was used for the functional inactivation of a constitutively expressed endogenous plant gene . Therefore, the following STNV hybrid vectors have been constructed:

pIF9 carrying the following operationally linked elements:

- a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811;
- a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 32 of SEQ ID No 3 (STNV-2 leader);
- a nucleic acid comprising the origin of assembly (OAS) of TMV-U1 from nucleotide 5443 to 5518 of SEQ ID No 2 or Genbank Accession Number V01408;
- a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948;
- a nucleic acid comprising the nucleotide sequence from nucleotide 806 to 1418 of SEQ ID No 3 (STNV-2 trailer).

pIF12 carrying the following operationally linked elements:

- a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811;
- a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 32 of SEQ ID No 3 (STNV-2 leader);
- a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948;
- a nucleic acid comprising the nucleotide sequence from nucleotide 742 to 1354 of SEQ ID No 3 (STNV-2 trailer).

Infective chimeric transcripts have been synthesized in vitro using T7 RNA polymerase with the linearized plasmid DNAs of the described pIF9 and pIF12 hybrid vectors as templates using standard procedures. Control in vitro transcripts have been synthesized on linearized plasmid DNAs of the precursor plasmids of pIF9 and pIF12 without the pds fragment inserts and on linearized plasmid DNA of an infective clone of the STNV wild type and of a hybrid STNV vector carrying an insert of the cat gene.

The in-vitro transcripts have been mechanically inoculated onto leaves of four weeks old Nicotiana benthamiana plants together with the TNV helper virus.

All infected plants were continuously scored for pds inactivation, which resulted in a phenotype showing leaf bleaching. For all inoculations necrotic lesions were observed for the inoculated leaves after 2 days post inoculation (p.i.). Within a week, necrotic lesions also occurred in upper leaves indicating the systemic spread of the viruses in N.benthamiana. In most infected plants, the virus symptoms have been severe but plants survived for many weeks.

Only plants, which have been infected with the hybrid vectors pIF9 and pIF12 carrying pds fragments, showed on top of the virus symptoms additional phenotypic changes. Approximately 4 weeks p.i., green upper leaves, which did not show any virus symptoms, developed bleached spots scattered all over the leaves. The bleaching was progressive and was not accompanied by necrotic lesions. Within another three weeks, the size of the spots increased constantly and the color changed from pale green to yellow to pale white-yellow. These symptoms have never been observed in plants, on which TNV/STNV wild type or TNV/STNV-deletion mutant control inoculations were carried out. Thus, this phenotype indicates the functional knockout of pds in N. benthamiana plants after infection with pIF9 and pIF12 hybrid viral vectors.

In RNA gel blot analyses performed with total RNA preparations of green and bleached spots of upper leaves of plants showing a FKO phenotype no chimeric STNV virus RNA and only very low levels of TNV helper virus RNA

could be detected. This indicates that virus induced gene silencing of pds in a specific tissue does not need to be accompanied by high levels of virus RNA.

Example 4: Inactivation of phytoene desaturase in Petunia hybrida using a TMV/STMV hybrid vector system.

A TMV/STMV hybrid vector system was used for the functional inactivation of a constitutively expressed endogenous plant gene. Therefore, the following STMV hybrid vectors have been constructed:

pVE293 carrying the following operationally linked elements:

- a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811
- a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 197 of STMV of SEQ ID No 4 or Genbank Accession Number M25782 (STMV leader)
- a nucleic acid comprising the origin of assembly (OAS) of TMV-U2 from nucleotide 5430 to 5505 of SEQ ID No 5 or Genbank Accession Number M34077 ("short" TMV OAS)
- a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948
- a nucleic acid comprising the nucleotide sequence from nucleotide 604 to 1058 of STMV of SEQ ID No 4 or Genbank Accesion Number M25782 (STMV trailer)
- a SP6 RNA polymerase promoter comprising nucleotide 143 to 124 of Genbank Accession Number X65308

pVE294 carrying the following operationally linked elements:

- a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811

- a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 197 of STMV of SEQ ID No 4 or Genbank Accession Number M25782 (STMV leader)
- a nucleic acid comprising the origin of assembly (OAS) of TMV-U2 from nucleotide 5441 to 5849 of SEQ ID No 2 or Genbank Accession Number M34077 ("long" TMV OAS)
- a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948
- a nucleic acid comprising the nucleotide sequence from nucleotide 604 to 1058 of STMV of SEQ ID No 4 or Genbank Accesion Number M25782 (STMV trailer)
- a SP6 RNA polymerase promoter comprising nucleotide 143 to 124 of Genbank Accession Number X65308

Infective chimeric transcripts have been synthesized in vitro using T7 RNA polymerase with the linearized plasmid DNAs of the described pVE293 and pVE294 hybrid vectors as templates using standard procedures. Control in vitro transcripts have been synthesized on linearized plasmid DNAs of the precursor plasmids of pVE293 and pVE294 without the pds fragment insert and on linearized plasmid DNA of the infective clone STMV-10 of the wild type.

The in vitro transcripts have been mechanically inoculated onto leaves of three months old Petunia hybrida V26 plants together with the TMV-U2 helper virus.

The infected plants have been continuously scored for pds inactivation, which resulted in a phenotype showing leaf bleaching. The infection of petunia plants with TMV/STMV caused the occurrence of crinkled leaves starting from the infected branches but quickly progressing systemically throughout the plant. The plants did survive the infections and often showed recovery phenotypes with almost no symptoms.

Only the plants, which have been infected with the hybrid vectors pVE293 and pVE294 carrying the pds fragment, showed in addition to the virus symptoms, additional phenotypic changes. In case of TMV/pVE293 infections, the infected branches developed young leaves with bleached sectors around the veins and at the tip of the leaves. This bleaching was progressive and independent from the presence of virus symptoms. Some of the leaves were fully bleached and progressively changed into almost white color. This phenotype was restricted to leaves of the infected branches. In case of TMV/pVE294 infections a similar bleaching of young leaves was observed for all developing branches but not for branches carrying already terminal flower buds. The bleaching started again around the veins and leaf tips but proceeded quickly producing variable leaf variegation patterns. This phenotype has never been observed in control inoculations with wild type viruses or deletion mutants. Therefore this phenotype indicates the functional knockout of pds in Petunia hybrida plants after infection with pVE293 and pVE294 viral hybrid vectors.

In RNA gel blot analyses performed with total RNA preparations of green and bleached spots of upper leaves of plants showing a FKO phenotype no chimeric STMV virus RNA and only low levels of TMV helper virus RNA could be detected. This indicates that virus induced gene silencing of pds in a specific tissue does not need to be accompanied by high levels of virus RNA.

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Claims

1. A method for isolating genes involved in the determination of a trait or phenotype of a plant species, said method comprising
   a.) Identifying a set of nucleic acid sequences of genes, whose expression is correlated with a trait of interest.
   b.) creating a library of gene silencing constructs in a viral RNA vector, said viral RNA vector being capable of replication inside plant cells and optionally movement between plant cells of a plant, and said gene silencing constructs being targeted to the nucleotide sequence of said nucleic acid sequences;
   c.) infecting a collection of individual plants of said plant species with said library of gene silencing constructs, whereby each plant is infected with at least one member of said library;
   d.) identifying a plant wherein said trait or phenotype is altered, using an assay adapted to said trait or phenotype;
   e.) isolating said gene involved in the determination of said trait or phenotype in said plant species, from said library based on the nucleotide sequence to which said gene silencing construct in said identified plant was targeted.

2. The method of claim 1, wherein said viral RNA vector is capable of autonomous replication inside plant cells and optionally autonomous movement between plant cells.

3. The method of claim 2, wherein said viral RNA vector is derived from cowpea mosaic virus.

4. The method of claim 1, wherein said viral RNA vector is capable of replication inside plant cells and optionally movement between plant cells when the required factors are supplemented *in trans*.

5. The method of claim 4, wherein said viral RNA vector is derived from a satellite RNA virus and said factors are supplemented by infection with a helper virus or helper virus RNA.

6. The method of claim 5, wherein said satellite RNA virus is satellite tobacco mosaic virus.

7. The method of claim 6, wherein said viral RNA vector comprises an origin of assembly of tobacco mosaic virus.

8. The method of claim 7, wherein said helper virus is tobacco mosaic virus.

9. The method of claim 5, wherein said satellite RNA virus is satellite tobacco necrosis virus.

10. The method of claim 9, wherein said viral RNA vector comprises an origin of assembly of tobacco mosaic virus.

11. The method of claim 10, wherein said helper virus is derived from tobacco necrosis virus and comprises a coat protein gene of tobacco mosaic virus and optionally a movement protein gene of tobacco mosaic virus.

12. The method of claim 11, wherein said satellite RNA virus is satellite tobacco necrosis vector strain 1 or 2 and said helper virus is derived from TNV-A.

13. The method of claim 11, wherein said satellite RNA virus is STNV-C and said helper virus is derived from TNV-D.

14. The method of claim 1, wherein said gene-silencing constructs comprise antisense RNA.

15. The method of claim 1, wherein said gene-silencing constructs comprise sense RNA.

16. The method of claim 1, wherein said gene-silencing constructs comprise an inverted repeat.

17. The method of claim 1, wherein said gene-silencing constructs comprise complementary stretch of at least 50 nucleotides of sense and antisense RNA.

18. The method of claim 17, wherein said gene-silencing constructs comprise complementary stretch of at least 100 nucleotides of sense and antisense RNA.

19. The method of claim 17, wherein said gene-silencing constructs comprise at least two copies of part of the nucleotide sequences of said collection of nucleic acids, said copies being in inverted repeat.

20. A method for the isolation of a nucleic acid with a specific function from a collection of nucleic acids, said collection of nucleic acids being characterized in that variation in the expression pattern of said nucleic acids is correlated with variation in a trait/phenotype of a plant harboring said nucleic acids, said method comprising the steps of

a.) creating a library of gene silencing constructs in a viral RNA vector, said viral RNA vector being capable of replication inside and optionally movement between plant cells, and said gene silencing constructs being targeted/adapted to the nucleotide sequence of said nucleic acids;

b.) infecting a collection of plants with said library of gene silencing constructs, whereby each plant is infected with at least one member of said library;

c.) identifying plants with altered trait or phenotype using an assay adapted to said trait or phenotype.

21. The method of claim 20, further comprising the step of isolating said nucleic acid with said specific function from said identified plant with altered trait or phenotype.

22. A method for determining the function encoded by a nucleic acid comprising a known nucleotide sequence in a plant, said method comprising

a.) providing a viral RNA vector, said viral RNA vector being derived from a satellite RNA virus, comprising a gene-silencing construct targeted to a gene comprising said known nucleotide sequence;

b.) infecting said plant with said viral RNA vector and a corresponding helper virus;

c.) identifying an altered trait or phenotype of said co-infected plant.

23. A method for isolating essential genes in a plant, comprising

a.) creating a library of random gene-silencing constructs for said plant comprised within a viral RNA vector, said viral RNA vector being derived from a satellite RNA virus;

b.) infecting a plant with at least one member of said library and with a corresponding helper virus;

c.) identifying plants developing a gene-silencing-construct-associated phenotype, preferably chlorosis or necrosis.

24. The method according to claim 23, further comprising the step of isolating the viral RNA vector from the tissue exhibiting the phenotype.

25. The method of claim 23, wherein said library is created by cloning random DNA fragments of said plant in a cDNA copy of the viral RNA vector.

26. The method of claim 23, wherein said library is created by cloning random cDNA fragments of said plant in a cDNA copy of the viral RNA vector.

27. The method of claim 23, wherein said library is created by cloning random duplicated cDNA fragments in inverted repeat.

28. The method of claim 23, wherein said viral RNA vector is derived from STMV and comprises an origin of assembly of tobacco mosaic virus, and wherein said helper virus is tobacco mosaic virus.

29. The method of claim 23, wherein said viral RNA vector is derived from satellite tobacco necrosis virus and comprises an origin of assembly of tobacco mosaic virus and wherein said helper virus is derived from tobacco necrosis virus and comprises a coat protein gene of tobacco mosaic virus and optionally a movement protein gene of tobacco mosaic virus.

30. The method of claim 29, wherein said satellite RNA virus is satellite tobacco necrosis vector strain 1 or 2 and said helper virus is derived from TNV-A.

31. The method of claim 29, wherein said satellite RNA virus is STNV-C and said helper virus is derived from TNV-D.

32. A method for the introduction of inhibitory RNA in the cytoplasm of plant cells, said method comprising:

a.) introducing into said plant cell, a viral RNA vector comprising said inhibitory RNA or comprising a chimeric nucleic acid which when transcribed yields said inhibitory RNA, wherein said viral RNA vector is derived from a satellite RNA virus; and

b.) introducing a corresponding helper virus into said plant cell,.

33. The method of claim 32, wherein said inhibitory RNA comprises sense RNA.

34. The method of claim 32, wherein said inhibitory RNA comprises antisense RNA.

35. The method of claim 32, wherein said inhibitory RNA comprises an inverted repeat.

36. The method of claim 32, wherein said inhibitory RNA comprises complementary stretch of at least 50 nucleotides of sense and antisense RNA.

37. The method of claim 36, wherein said inhibitory RNA comprises complementary stretch of at least 100 nucleotides of sense and antisense RNA.

38. The method of claim 32, wherein said viral RNA vector is derived from STMV and comprises an origin of assembly of tobacco mosaic virus, and wherein said helper virus is tobacco mosaic virus.

39. The method of claim 32, wherein said viral RNA vector is derived from satellite tobacco necrosis virus and comprises an origin of assembly of tobacco mosaic virus and wherein said helper virus is derived from tobacco necrosis virus and comprises a coat protein gene of tobacco mosaic virus and optionally a movement protein gene of tobacco mosaic virus.

40. The method of claim 39, wherein said satellite RNA virus is satellite tobacco necrosis vector strain 1 or 2 and said helper virus is derived from TNV-A.

41. The method of claim 39, wherein said satellite RNA virus is STNV-C and said helper virus is derived from TNV-D.

42. The method of any one of claims 32 to 41, wherein said plant is selected from Nicotina spp, Oryza sativa, Zea Mays, Brassica spp. , Gossypum spp., Triticum spp., Arabidopsis spp. or Petunia spp.

43. A kit for introduction of inhibitory RNA in the cytoplasm of a plant cell, said kit comprising

a.) a viral RNA vector derived from a satellite RNA virus, said vector comprising a chimeric nucleic acid which when transcribed yields said inhibitory RNA or which comprises said inhibitory RNA; and

b.) a corresponding helper virus.

44. The kit of claim 43, wherein said inhibitory RNA comprises sense RNA.

45. The kit of claim 43, wherein said inhibitory RNA comprises antisense RNA.

46. The kit of claim 43, wherein said inhibitory RNA comprises an inverted repeat.

47. The kit of claim 43, wherein said inhibitory RNA comprises complementary stretch of at least 50 nucleotides of sense and antisense RNA.

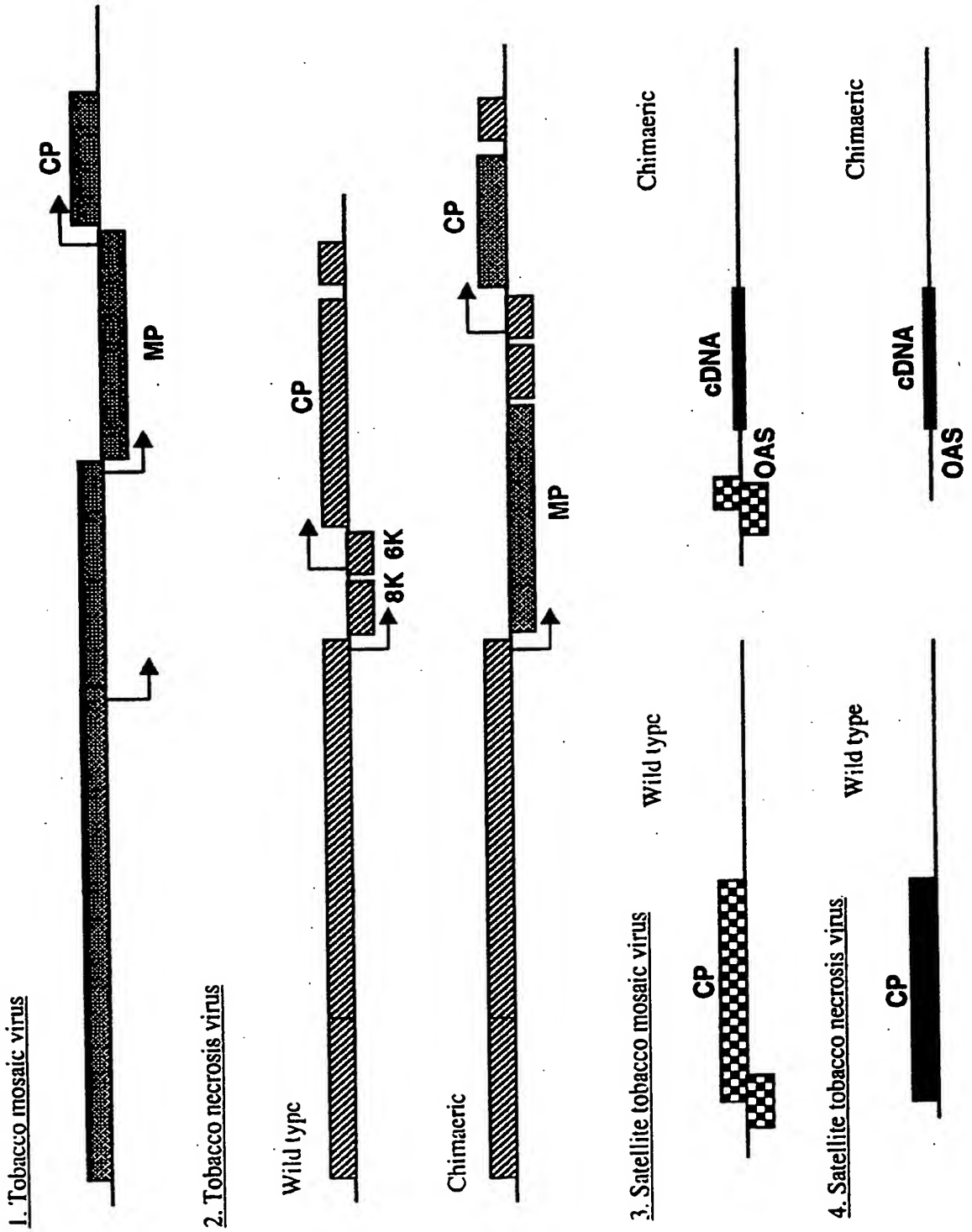
48. The kit of claim 43, wherein said inhibitory RNA comprises complementary stretch of at least 100 nucleotides of sense and antisense RNA.

49. The kit of claim 43, wherein said viral RNA vector is derived from STMV and comprises an origin of assembly of tobacco mosaic virus, and wherein said corresponding helper virus is tobacco mosaic virus.

50. The kit of claim 43, wherein said viral RNA vector is derived from satellite tobacco necrosis virus and comprises an origin of assembly of tobacco mosaic virus and wherein said corresponding helper virus is derived from tobacco necrosis virus and comprises the coat protein gene of tobacco mosaic virus and optionally the movement protein gene of tobacco mosaic virus.

51. The kit of claim 50, wherein said satellite RNA virus is satellite tobacco necrosis vector strain 1 or 2 and said corresponding helper virus is derived from TNV-A.

52. The kit of claim 50, wherein said satellite RNA virus is STNV-C and said corresponding helper virus is derived from TNV-D.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atcgcgcggg tcaaatgtat atggttcata tacatccgca ggcacgtaat aaagcgaggg 6360
gttcgaatcc ccccgttacc cccggtaggg gccca 6395

<210> 3
<211> 1245
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA copy of
the nucleotide sequence of the genome of STNV-2

<400> 3
agtaaagaca ggaaacttta ccgactatca gaatgacaaa acgtcaaagc aaacaatcaa 60
accgcaagag cgttgcatca caggtgcgta gtattgttga gtcaatggct gagcagaagc 120
gatttgcttt tcttacgaac accaacacag tcactacagc aggtaccgtg atcaacctga 180
gcaacaacat cgtgcaagga gatgaccttg ttaatcgcac cggagaccag attaagacca 240
tacaccagac tttattgact cggtgtacag gaattaccaa cagccaaagc tttcggttca 300
tctggtttcg tgacaacacc aataggggga ctacaccggc tgtgactgag gtgttagaca 360
gtgctagtat aacatcccag tataacccca ctacgttcca gcaaaagagg ttcactgttt 420
tccaagattt catgttggat acctctatag ttggacgtgt gattgtccat cggactgccg 480
ttgataagaa acggcgtgcg atattttaca acggtgctgc ttctgtagcc gcgtcaaatg 540
gccccggtgc cacatttgta cttgtcattg gatcacatgc cactggacag tatgatgtga 600
cagccgagat tgtttatctg gacatgtaga ccatggtcat gatgatgata gtgaaggacg 660
ctgaaagatg cgtagctacc ctcctggtgc acttcctggt gcaaagcaga accaaagggt 720
acggtggtac ggcggacagt agtcctgaac tagtaaatca ggaccgggag aaaaccagct 780
gacggctaaa tccattccca ctagtgtatt agtggaacga ggccccgcgt gaattggggt 840
ggctgcatgg ggtggaaaac catgtggtcg cagtcatttc tcctatgcat tattgtctca 900
atacttgtgt gcaacaatgc tgttaatcaa cgtagcactc aacatcactt caaaaccccc 960
tccatgtcac aagaatcaag atgcatgtct gtgtttagcg gtatatattt tgcatccact 1020
tgatcgtgat tttgccctgg gcacctcgcg cggttggtac ccgcggagac tccccacagc 1080
aacatggcat taggcaggga taaggtatag tgactagaca aatgcgcgtg aagctggaaa 1140
gtccggttag cagtggggtt gtgcggaatg cagcctcaac aaggtatagc tgctgcatag 1200

gagatgtgaa cctttcaaac ttgaattcaa gtctcatgac tgccc 1245

<210> 4
<211> 1058
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA copy of the nucleotide sequence of the genome of STMV

<400> 4
agtaaactta ccaatcaaaa gacctaacca acaggactgt cgtggtcatt tatgctgttg 60
ggggacatag ggggaaaaca tattgccttc ttctacaaga ggccttcagt cgccataatt 120
acttggcgcc caattttggg tttcagttgc tgtttccagc tatggggaga ggtaaggtta 180
aaccaaaccg taaatcgacg ggtgacaatt cgaatgttgt tactatgatt agagctggaa 240
gctatcctaa ggtcaatccg actccaacgt gggtcagagc catacctttc gaagtgtcag 300
ttcaatctgg tattgctttt aaagtaccgg tcgggtcact attttcggca aatttccgga 360
cagattcctt tacaagcgtc acagtgatga gtgtccgtgc ttggacccag ttaacaccgc 420
cagtaaatga gtacagtttt gtgaggctga agccattgtt caagactggt gactctactg 480
aggagttcga agggcgtgca tcaaacatca acacacgagc ttctgtaggg tacaggattc 540
caactaattt gcgtcagaat actgtggcag ccgacaatgt atgcgaagta agaagcaact 600
gtcgacaagt cgccttggtt atttcgtgtt gttttaactg aacctcgaca taagcctttt 660
ggatcgaagg ttaaacgatc cgctcctcgc ttgagcttga ggcggcgtat ctcttatgtc 720
aacagagaca ctttggtcta tggttgtata acaatagata gactcccgtt tgcaagatta 780
gggttaacag atcttgccgt tagtctggtt agcgcgtaac cggccttgat ttatggaata 840
gatccattgt ccaatggctt tgccaatgga acgccgacgt ggctgtataa tacgtcgttg 900
acaagtacga aatcttgtta gtgtttttcc ctccacttaa atcgaagggt tttgttttgg 960
tcttcccgaa cgcatacgtt agtgtgacta ccgttgttcg aaacaagtaa aacaggaagg 1020
gggttcgaat ccctccctaa ccgcgggtaa gcggccca 1058

<210> 5
<211> 6355
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA copy of the nucleotide sequence of the genome of TMV-U2

<400> 5
gatgttttaa tagttttcga caacaacaat taaaacaaaa acaacatatt acaaacaaca 60
aacaacaaca atggcacaca tacaatctat aattagcaac gcccttcttg aaagcgtgag 120
tggtaaaaac actctcgtta atgaccttgc aagaaggcgc atgtacgata cggccgtgga 180
agaatttaac gcccgcgacc gtagaccaaa ggtcaacttt tccaaaacta ttagcgaaga 240
gcaaacgctt ctagtctcca acgcgtaccc ggagttccag attacctttt ataatactca 300
aaatgccgta cacagtttgg ctggaggttt gagagcatta gaattggaat atctgatgct 360
acaagttccc tatggatcgc cgacatatga tataggtggg aactttgcag cacatttgtt 420

caaaggcagg gattacgtgc attgctgtat gcccaatctg gacatacgag atataatgag 480
gcacgaagga caaaaggact caattgagat gtatttgtcc agattgtctc gttctaacaa 540
ggtaattcct gagtttcaaa gggaggcttt taacaggtat gcagaagctc ccaacgaagt 600
ctgctgctct aaaacttttc aggattgtcg aatacatccg ccagagaata gtggtagaag 660
atacgctgtt gctctgcaca gtttgtatga tattcctgtg catgagtttg gagctgcgtt 720
aatatctaag aatatacatg tatgttatgc agcttccatt ttggcagaag cattattact 780
agaccagacg gaggttacgc ttaatgaaat aggcgcaact ttcaaaagag aaggtgatga 840
tgtttctttt ttctttgctg atgaaagtac tttaaattat agtcataaat acaaaaatat 900
cttgcattat gtagttaaat cttactttcc tgcttctagt agaatagttt actttaagga 960
atttttagtc actagggtta atacttggtt ttgtaaattt accaaagtag atacctatat 1020
tctgtacaag agtgttagac aagtagggtg tgatagtgat cagttctatg aggcgatgga 1080
agacgccttt gcttacaaga aaaccttggc catgttcaac actgaaagag caatctttag 1140
agacacggct tcggttaact tttggttccc taagatgaag gacatggtga tagtaccgct 1200
gtttgagggt tctattacca gcaaaaagat gacaaggagt gaggtcattg ttaatcgtga 1260
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gatcaagatc ccagatctgt atgtcacatg gaaagacagg ttcgtagctg aatacaccaa 1680
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cgacgcgtta tcagaattat ctatccttaa gggtgctgat aatttcgata tcgcgaagtt 1800
caaagacatg tgcaaggctt tagatgttag tcctgatgtg gcagcacgag taatcgttgc 1860
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agaggtgaac gtaaataaat tttctattgc tgagaaaggg agattgcctg tgtgtgcaga 2040
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ccataaggct tgcgtggata gtgtgattac aaagcaaatg gcatcggttg tctacactgg 2160
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actggttcgt ggaactggca tgttcaatca agcaggcttt gagactgcta gtggacttgt 6120
ctggaccaca actccggcta cttagctatt gttgtgagat ttcctaaaat aaagtcgctg 6180

aagacttaaa attcagggtg gctgatacca aaatcagcag tggttgttcg tccacttaaa 6240
tataacgatt gtcatatctg gatccaacag ttaaaccatg tgatggtgta tactgtggta 6300
tggcgtaaaa catcggagag gttcgaatcc tcccctaacc gccggtagcg gccca 6355

<210> 6
<211> 2346
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequenc : nucleotide
sequence of the tomato phytoene desaturase (pds)
encoding cDNA

<400> 6
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acaagtttcc atttaactct tcaacttcaa cccaaccaaa tttatttcct taattgtgca 120
gaaccactcc ctatatcttc taggtgcttt cattcgttcc gaggtaagaa aagatttttg 180
tttctttgaa tgctttatgc cactcgttta acttctgagg tttgtggatc ttttaggcga 240
ctttttttt ttttgtatgt aaaatttgtt tcataaatgc ttctcaacat aaatcttgac 300
aaagagaagg aattttacca agtatttagg ttcagaaatg gataattttc ttactgtgaa 360
atatccttat ggcaggtttt actgttattt ttcagtaaaa tgcctcaaat tggacttgtt 420
tctgctgtta acttgagagt ccaaggtagt tcagcttatc tttggagctc gaggtcgtct 480
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agcgaatcaa tgggtcataa gttaaagatt cgtactcccc atgccacgac cagaagattg 600
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ccaactaaac cattggagat tgttattgct ggtgcaggtt tgggtggttt gtctacagca 780
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ttctttgggg cttacccaaa tattcagaac ctgtttggag aattagggat taacgatcga 960
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cctgatgaaa tttcagcaga tcaaagcaaa gcaaaaatat tgaagtacca tgttgtcaaa 1920
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tccccaatag aggggtttta tttagccggt gactacacga aacagaaata cttggcttca 2040
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ttacttgttg gacgtagcca aaagaagttg tcggaagcaa gcgtagttta gctttgtggt 2160
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aagaagagtg cgaggtgaag caagtaggag aaatgttagg aaagctccta tacaaaagga 2280
tggcatgttg aagattagca tctttttaat cccaagttta aatataaagc atattttatg 2340
gaattc 2346

<210> 7
<211> 7096
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: nucleotide sequence of the tobacco nitrate reductase (nia-2) encoding cDNA

<400> 7
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gaggacctta acttgtttgt ctttgttgct ttctgcaaat ttcaccttaa cagcccattt 120
gagattgatt tagttagtta taacaattag ttaaatgctt gtgtaatttg aagaaaatat 180
ttggacgtgc tcgctgaaaa cattatactc ctatataata gaaatacttt ctgaaaagtt 240
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tacatataca tataacctta ctttttgatt actaaaaatt taattatatt taatttgggt 480
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tatgtttagc ttgggtaaat atcagatgtc actaaacatt ttacctagcc attcctccga 600
aaagaaattg agaaggaaat tagagttagt ggagccataa taatgtttaa tgtgaccata 660
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gcgtaaaaag tgtaaatcgt tagaattgtt ttatttattt tatgatgtca ctatttttta 840
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ggttaaacaa accattggtt tcaactgggg cgccgctgcc gtttcaacaa cgatatggcg 1740

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gaaagggaaa tttcttagtt catggcaaac agaagtttgc caagaagttg gccatgatag 6120
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cgaaagttgt aatgtttacc atgacaaatt gtattcaatt cctcatggaa tagtaacatt 6780
gtgttcatgt gtcttcctgt aagcgatctt caaaatatca atgtatatat atagtaattg 6840
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aaaccacgaa tatcattatg cagacgtata ggtaattaat ctacttttttg aaaaaaaatc 7020
tattttcttt atgtggtcct tcaaaataat attctagaac cttttgtata ttccctttta 7080
acttctattt agtttt 7096

<210> 8
<211> 1839
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: nucleotide sequence of the tobacco nitrite reductase (nir-1) encoding cDNA

<400> 8
tttctattaa atttctggca ccttcattgc caaatccagc tagattttcc aagaatgctg 60
tcaagctcca cgcaactccg ccgtctgtgg cagcgccgcc agctggtgct ccagaggttg 120
ctgctgagag gctagaaccc agagttgagg aaaaagatgg ttattggata ctcaaggagc 180
agtttagaaa aggcataaat cctcaagaaa aggtcaagat tgagaagcaa cctatgaagt 240
tgttcatgga aaatggtatt gaagagcttg ctaagatacc cattgaagag atagatcagt 300
ccaagcttac taaggatgat attgatgtta ggcttaagtg gcttggcctc ttccatagga 360
gaaagaacca atatgggcgg ttcatgatga gattgaagct tccaaatgga gtaacaacga 420
gtgcacagac tcgatacttg gcgagtgtga taaggaaata cgggaaagaa ggatgtgctg 480
atattacaac gaggcaaaat tggcagattc gtggagttgt actgcctgat gtgcccgaga 540
tactaaaggg actagcagaa gttgggttga ccagtttgca gagtggcatg gacaatgtca 600
ggaatccagt aggaaatcct cttgctggaa ttgatccaga agaaatagta gacacagggc 660
cttacactaa tttgctctcc caatttatca ctggcaattc acgaggcaat cccgcagttt 720
ctaacttgcc aaggaagtgg aatccgtgcg tagtaggctc tcatgatctt tatgaacatc 780
cccatatcaa cgatctcgcg tacatgcctg ccacgaaaga tggacgattt ggattcaacc 840
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caggctgccc gaatacgtgt gcacaagttc aagttgcgga cattggattc atgggatgcc 1560
tgactagaga taagaatgga aagactgtgg aaggcgccga tgttttctta ggaggcagaa 1620
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taccacttgt tgtggactta ctagttaaca actttggtgc agttccacga gaaagagaag 1740
aaacagaaga ctaataaaat ttagaatagt tggtgatttt gctgtgttca taacatgtaa 1800
tgtatgataa atcaatgcaa acatttctac ctacgtgag 1839

<210> 9
<211> 1294
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA of the beta-1,3-glucanase of Nicotiana plumbagenifolia

<400> 9

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taggagctca atcagtaggt gtttgctacg gaatgctggg caacaacttg ccaccagcat 120
cacaagttgt acaactgtac aagtcaaaaa acataagaag aatgaggctt tatgatccaa 180
atcaagcagc tttacaggct ttaagaggct ccaacattga agttatgtta ggagttccca 240
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atgtcagaaa tttctggcca gccgttaaat ttaggtacat tgccgttgga aatgaagtca 360
gccctgtaac aggcacatct tcacttaccc gatatcttct tccggccatg aggaacattc 420
ggaatgcgat ttcttcagca ggtttgcaaa acaatatcaa agtctcaagt tctgtagaca 480
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caatgtcgga tgctgtgtat gctgccctgt ctcgagccgg agggggctcg atagagattg 780
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caacttacta caagaactta attcagcatg ttaaaagggg tagtccaaga aggcctaata 900
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gtgagatgtg ataagagagt tctctttaaa tatctttaca tggatggaaa acttagtacc 1140
aataactaga ttgtttcttt ctttatgcaa ttttcttgta atgagagact agtacttgct 1200
ctctgtgtcc ttgtggagag taactagaga caaattaagc aaataacata aataattgag 1260
tgttgattct gcaatgataa atagaaaaaa aaaa 1294

<210> 10
<211> 720
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: green
fluorescent protein encoding regon

<400> 10
atggtgagca agggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac 60
ggcgacgtaa acggccacaa gttcagcgtg tccggcgagg gcgagggcga tgccacctac 120
ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggccccac 180
ctcgtgacca ccctgaccta cggcgtgcag tgcttcagcc gctaccccga ccacatgaag 240
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ttcaaggacg acggcaacta caagacccgc gccgaggtga agttcgaggg cgacaccctg 360
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aagctggagt acaactacaa cagccacaac gtctatatca tggccgacaa gcagaagaac 480
ggcatcaagg tgaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc 540
gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac 600
tacctgagca cccagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc 660
ctgctggagt tcgtgaccgc cgccgggatc actctcggca tggacgagct gtacaagtaa 720

<210> 11
<211> 1809

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial
Sequence:beta-glucuronidase encoding region

<400> 11
atggtccgtc ctgtagaaac cccaacccgt gaaatcaaaa aactcgacgg cctgtgggca 60
ttcagtctgg atcgcgaaaa ctgtggaatt gatcagcgtt ggtgggaaag cgcgttacaa 120
gaaagccggg caattgctgt gccaggcagt tttaacgatc agttcgccga tgcagatatt 180
cgtaattatg cgggcaacgt ctggtatcag cgcgaagtct ttataccgaa aggttgggca 240
ggccagcgta tcgtgctgcg tttcgatgcg gtcactcatt acggcaaagt gtgggtcaat 300
aatcaggaag tgatggagca tcagggcggc tatacgccat ttgaagccga tgtcacgccg 360
tatgttattg ccgggaaaag tgtacgtatc accgtttgtg tgaacaacga actgaactgg 420
cagactatcc cgccgggaat ggtgattacc gacgaaaacg gcaagaaaaa gcagtcttac 480
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aacacctggg tggacgatat caccgtggtg acgcatgtcg cgcaagactg taaccacgcg 600
tctgttgact ggcaggtggt ggccaatggt gatgtcagcg ttgaactgcg tgatgcggat 660
caacaggtgg ttgcaactgg acaaggcact agcgggactt tgcaagtggt gaatccgcac 720
ctctggcaac cgggtgaagg ttatctctat gaactgtgcg tcacagccaa aagccagaca 780
gagtgtgata tctacccgct tcgcgtcggc atccggtcag tggcagtgaa gggcgaacag 840
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aaccacccaa gcgtggtgat gtggagtatt gccaacgaac cggatacccg tccgcaagtg 1260
cacgggaata tttcgccact ggcggaagca acgcgtaaac tcgacccgac gcgtccgatc 1320
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gtgctgtgcc tgaaccgtta ttacggatgg tatgtccaaa gcggcgattt ggaaacggca 1440
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atcaccgaat acggcgtgga tacgttagcc gggctgcact caatgtacac cgacatgtgg 1560
agtgaagagt atcagtgtgc atggctggat atgtatcacc gcgtctttga tcgcgtcagc 1620
gccgtcgtcg gtgaacaggt atggaatttc gccgattttg cgacctcgca aggcatattg 1680
cgcgttggcg gtaacaagaa agggatcttc actcgcgacc gcaaaccgaa gtcggcggct 1740
tttctgctgc aaaaacgctg gactggcatg aacttcggtg aaaaaccgca gcagggaggc 1800
aaacaatga 1809

<210> 12
<211> 411
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA copy of
part of the region of a TMV-U2 variant comprising

the origin of assembly

<400> 12
ccctcgccaa ttgaactcac tgaaaaagtt gttgatgagt tcgtagatga agtaccgatg 60
gctgtgaaac tcgaaaggtt ccggaaaaca aaaaagagag tggtaggtaa taatgttaat 120
aataagaaaa taaataatag tggtaagaag ggtttgaaag ttgaggaaat tgaggataat 180
gtaagtgatg acgagtctat cgcgtcatcg agtacgtttt aatcaatatg ccttatacaa 240
tcaactctcc gagccaattt gtttacttaa gttccgctta tgcagatcct gtgcagctga 300
tcaatctgtg tacaaatgca ttaggtaacc agtttcaaac gcaacaagct aggacaacag 360
tccaacagca atttgcggat gcctggaaac ctgtgcctag tatgacagtg a 411

<210> 13
<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA copy of
STMV leader region

<400> 13
agtaaaactt accaatcaaa agacctaacc aacaggactg tcgtggtcat ttatgctgtt 60
gggggacata gggggaaaac atattgcctt cttctacaag aggccttcag tcgccataat 120
tacttggcgc ccaattttgg gtttcagttg ctgtttccag ctatggggag aggtaaggtt 180
aaaccaaacc gtaaatcg 198

<210> 14
<211> 455
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:cDNA copy of
STMV trailer region

<400> 14
gacaagtcgc cttggttatt tcgtgttgtt ttaactgaac ctcgacataa gccttttgga 60
tcgaaggtta aacgatccgc tcctcgcttg agcttgaggc ggcgtatctc ttatgtcaac 120
agagacactt tggtctatgg ttgtataaca atagatagac tcccgtttgc aagattaggg 180
ttaacagatc ttgccgttag tctggttagc gcgtaaccgg ccttgattta tggaatagat 240
ccattgtcca atggctttgc caatggaacg ccgacgtggc tgtataatac gtcgttgaca 300
agtacgaaat cttgttagtg ttttttcctc cacttaaatc gaagggtttt gttttggtct 360
tcccgaacgc atacgttagt gtgactaccg ttgttcgaaa caagtaaaac aggaaggggg 420
ttcgaatccc tccctaaccg cgggtaagcg gccca 455

<210> 15
<211> 1971
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA copy of part of the genome of a TMV-U1 variant, comprising MP and CP genes

<400> 15
ggaaacactg tgattatagc tgcatgtttg gcctcgatgc ttccgatgga gaaaataatc 60
aaaggagcct tttgtggtga cgatagtctg ctgtacttcc caaagggttg tgagtttccg 120
gatgtgcaac actccgcgaa tcttatgtgg aattttgaag caaaactgtt taaaaaacag 180
tatggatact tttgcggaag gtatgtaata catcacgaca gaggatgcat tgtgtattac 240
gatcccctaa agttgatctc gaaacttggt gctaaacaca tcaaggattg ggaacacttg 300
gaggagttca gaaggtctct ttgtgatgtt gctgtttcgt tgaacaattg tgcgtattac 360
acacagttgg acgacgctgt atgggaggtt cataagaccg cccctccagg ttcgtttgtt 420
tataaaagtc tggtgaagta tttgtctgat aaagttcttt ttagaagttt gtttatagat 480
ggctctagtt gttaaaggaa aagtgaatat caatgagttt atcgacctga caaaaatgga 540
gaagatctta ccgtcgatgt ttacccctgt aaagagtgtc atgtgttcca aagttgataa 600
aataatggtt catgagaatg agtcattgtc agaggtaaac cttctcaaag gagttaagct 660
tattgatagt ggatacgtct gtttagccgg tttggtcgtc acgggcgagt ggaacttgcc 720
tgacaattgc agaggaggtg tgagcgtgtg tctggtggac aaaaggatgg aaagagccga 780
cgaggccact ctcggatctt actacacagc agctgcaaag aaaagatttc agttcaaggt 840
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caatattaga aatgtaaaga tgtcagcggg tttctgtccg ctttctctgg agtttgtgtc 960
ggtgtgtatc gtttatagaa ataatataaa attaggtttg agagagaaga tcacaagtgt 1020
gagagatgga gggcccatgg aacttacaga agaagttgtt gatgagttca tggaagatgt 1080
ccctatgtca atcaggcttg caaagtttcg atctcgaacc ggaaaaaaga gtgatgtccg 1140
taaagggaaa attagtagta gtgatcggtc agcgccgaac aagaactata gaaatgttaa 1200
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